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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
Katsuo KUMAGAI et al.	)
Serial No. **10/629,117**	) Art Unit: **Unassigned**
Filed: **July 28, 2003**	) Examiner: **Unassigned**
For: **THERAPEUTIC AGENT FOR MASTITIS OF LIVESTOCK AND METHOD FOR TREATING MASTITIS USING THE SAME AGENT**	)

DECLARATION UNDER 37 C.F.R. 1.132 BY KENZO KAI

Commissioner for Patents
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I, Kenzo Kai, declare as follows:

1. I received a Doctor's Degree in Agriculture from Tohoku University in 2002. I have been employed as a researcher in T-Cell Research Institute for at least seven years.

2. I am one of the inventors of the invention claimed in the above-referenced application.

3. Under my direction, the following experiment was performed to compare the cytotoxic activity of the claimed compound, glycyrrhizin (GL) to that of glycyrrhetinic acid mono-glucuronide (GAMG) and glycyrrhetinic acid (GA). The minimum cytotoxic concentration of GL was determined to be approximately 2,000 μg/ml, whereas the minimum cytotoxic concentration of GAMG was determined to be 250 μg/ml. These results suggest that, for bovine mammary epithelial cells (BMEC), GAMG is 8-fold more toxic than GL.

Experiment

BMEC were treated separately with each of the following three compounds: GL, GAMG and GA. The compounds were all kindly obtained from Minophagen Pharmaceutical (Japan) as monoammonium salt bound compounds ($\geq$ 95% purity). The three compounds were dissolved in phosphate-buffered saline, pH 7.2 at 100

mg/ml, and adjusted to a pH of 7.0. These solutions were preserved at –80℃ until used.

Cell Culture

BMEC were kindly obtained from Dr. Aso at Tohoku University in Japan. Details of BMEC can be found in Rose, M.T. *et al., J. Dairy Res.* 2002; 69:345-355, abstract attached. The BMEC were maintained as monolayer cultures at 37℃ in Dulbecco's modified Eagle medium (Gibco, U.S.A.) supplemented with 20% heat-inactivated fetal bovine serum, 10 μg/ml transferrin, 5 mM sodium acetate in a humidified 5% CO_2 atmosphere.

Assay for Cytotoxic Activity

To determine cytotoxic activity for GL, GAMG and GA, the cells were transferred to a 96-microwell plate (Becton Dickinson, U.S.A.) at 5 x 10^3 cells/200 μl per well. To adhere to the plate, the cells were incubated for 8 hours at 37°C. After incubation, the cell culture medium was replaced with a serum-free medium, S-Clone SF-H (Sanko Jyunyaku, Japan), and incubated for 17 hours at 37°C.

It is known that lactate dehydrogenase (LDH) is released from the cytosol of damaged cells, but not viable cells (live cells). The concentration of LDH in culture medium was measured using a commercial cytotoxicity detection LDH kit (Roche Molecular Biochemicals, Germany), a colorimetric assay for the quantification of cell damage. The amount of enzymatic activity in the culture supernatant directly correlates to the amount of formazan formed during the reaction (in this case 30 minutes). After the reaction of LDH, the intensity of red formazan dye was determined by spectrophotometric absorbance at 490 nm with SPECTRAmax 250 (Molecular Devices, U.S.A.) and analyzed by SOFTmax PRO1.1 (Molecular Devices, U.S.A.).

The percentage of compound-mediated cytotoxicity was calculated as follows:

Cytotoxicity (%) = 100 × [(value of experiment well) – (value without compounds)] / [(value of 100 % cell-lysis) - (value without compounds)]

The absorbance at 490 nm for the value of 100% cell-lysis was 0.49.

The absorbance at 490 nm for the value of well without compounds was 0.03.

Results

The 50% cytotoxic concentration of GAMG was determined to be approximately 2,000 μg/ml. Due to the low toxicity of compound GL, a 50%

cytotoxic concentration was never reached in the present experiment. The minimum cytotoxic concentration of GL and GAMG were 2,000 μg/ml and 250 μg/ml (or less), respectively. These results demonstrate that cytotoxic activity of GAMG in BMEC is at least 8-fold higher than GL.

Cytotoxicity of GL, GAMG, and GA in Bovine Mammary Epithelial Cells			
concentration of compounds (μ g/ml)	Tested compounds (incubation for 17 hours)		
	GL	GAMG	GA
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
31.25	0.0 ± 0.0	0.0 ± 0.0	8.2 ± 1.1
62.5	0.0 ± 0.0	0.0 ± 0.0	18.4 ± 2.6
125	0.0 ± 0.0	0.4 ± 0.1	26.5 ± 7.1
250	0.0 ± 0.0	6.1 ± 0.4	38.8 ± 1.0
500	0.0 ± 0.0	30.6 ± 2.6	55.1 ± 0.3
1,000	0.0 ± 0.0	38.8 ± 4.1	67.3 ± 5.0
2,000	12.2 ± 1.2	51.0 ± 2.0	97.0 ± 0.2
4,000	26.5 ± 3.0	91.8 ± 3.0	98.0 ± 1.0
Cytotoxicity (%) = 100×[(value of experiment) - (value of without compounds)] / [(value of well with 100% cell-lysis) - (value of well without compounds)]			

Conclusion

The results of this experiment demonstrate that GA is approximately four times more cytotoxic than GAMG, which is at least eight times more toxic than the claimed compound, GL. GA exhibits 50% cytotoxicity at a concentration of approximately 500 μg/ml and GAMG displays 50% cytotoxicity at a concentration of approximately 2,000 μg/ml. Unexpectedly, GL fails to reach 50% cytotoxicity at the concentrations of compound used in this experiment. A mere 26% cytotoxicity is observed at the maximum concentration used in this experiment, 4,000 μg/ml.

By exhibiting this surprisingly low level of cytotoxicity, GL may play a significant role in the treatment of mastitis in livestock. For example, if GAMG or GA were used to treat mastitis, the duration of treatment would need to be limited or the dosage reduced to avoid adverse effects caused by the toxicity of these compounds. In contrast, GL is a superior drug candidate for mastitis treatment because high doses or prolonged treatment can be utilized, thereby resulting in a faster recovery. Furthermore, GL may be safely used to prevent a persistent firmness

of infected mammary gland and to recover an injured mammary gland in livestock as soon as possible. An increase in milk production by reducing recovery time is of considerable economic importance to the dairy industry.

I am aware of the publication of *Toshimitsu et al.,* (JP 06-305932) which describes the use of GAMG as an active ingredient in a topical preparation. It is my opinion that, due to its cytotoxicity GAMG is unsuitable for direct administration to the mammae or for the treatment of mastitis as claimed in the above-identified patent application.

Furthermore, given the structural similarity of the GL, GAMG and GA compounds, one would not anticipate significant differences in cytotoxicity. Therefore, one skilled in the art would not be motivated to use **any** of these compounds for the treatment of mastitis. The inventors of the above-identified application **unexpectedly** discovered that GL could be used for treating mastitis with minimal risk of adverse effects.

14 January 200X
Date

Kenzo Kai
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